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The breast produces inhibitors of breast tumor formation. We hypothesized increases the amount of these compounds would delay cancer onset. We study the molecule TGF- β which blocks cell growth. TGF- β is produced as latent TGF-β complex consisting of the TGF-β homodimer, the TGF-β propeptide dimer, and a second gene product, the latent TGF-β binding protein (LTBP). LTBP targets latent TGF-β to the extracellular matrix, from which active TGF- β is released. The third cysteine rich repeat (CR3) of LTBP-1 is necessary and sufficient for covalent interaction with small latent TGF- β. CR3 overexpression should result in the TGF- β propeptide complexing to an LTBP form unable to interact with matrix. Therefore, TGF-β in this complex should be more easily activated. We generated mice overexpressing CR3 under the control of breast specific WAP promotor, and will generate mice overexpressing CR3 under the control of MMTV LTR. We will study whether breast cancer occurrence is delayed compared to wt animals. We will test whether tamoxifen treatment, which prevents breast cancer, and overproduction of TGF β in genetically engineered mice block tumorigenesis better than either condition alone.

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Branka Jaborc 7/26/2000

PI - Signature Date

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Introduction

TGF-β is the most potent inhibitor of the progression of normal mammary epithelial cells through the cell cycle (Robinson et al. 1991). During the early stages of breast cancer development, the transformed epithelial cells are sensitive to TGF-β-mediated growth arrest, and TGF-β acts as an anti-tumor agent. We hypothesized that, if methods were found that increased the amount of naturally produced TGF-β, the onset of cancer might be delayed or prevented. TGF-β is normally produced in an inactive form and must be freed from the inactive complex to be active. The latent TGF-β complex consists of the TGF-β homodimer plus it's N-terminal precursor propeptide dimer and is named small latent complex (SLC) (Derynck et al. 1985; McMahon et al.1996; Miller et al. 1992). The precursor propeptides are disulfide bonded to a second gene product, the latent TGF-β binding protein (LTBP) (Kanazaki et al. 1993), and this complex is called the large latent complex (LLC). Most cells (such as T47-D breast cell carcinoma cells) produce TGF-β as part of the LLC (Miyazono et al. 1993; Sporn et al. 1992; Masague et al. 1992; Harpel, 1998). LTBP targets latent TGF-β to the extracellular matrix, from which biologically active TGF-β may be released. Our lab demonstrated that the third cysteine rich repeat (CR3) of LTBP-1 is necessary and sufficient for covalent interaction with small latent TGF-β (Gleizes et al. 1996). We reasoned that overexpression of this domain, CR3, should result in all of the TGF-β propeptide c omplexing to an LTBP1 form that is unable to interact with matrix and therefore would be more easily activated. I proposed to generate genetically engineered mice that express CR3 under the control of breast-specific MMTV and WAP promotors. This should increase the local concentration of TGFβ in mammary tissue and supress mammary tumor formation. As the anti cancer agent tamoxifen is believed to enhance latent TGF-β activation, we also proposed to determine if tamoxifen acted synergistically in delaying tumors in transgene vs. normal mice.

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For the first year of this project, we proposed to construct the CR3 transgenes, test the expression of the transgenes in cultured cells, create transgenic mice that express each of two transgenes, and establish lines of mice that express high levels of each transgene. Even though the initiation of the project was delayed because of difficulties obtaining the WAP and MMTV promoter constructs, we have achieved most of our stated goals.

The intent of the proposal was to create a latent complex of TGF-β that was more easily activated than the normal complex. The latent TGF-β complex consists of TGF-β, the TGF-β propeptides, which remain associated with TGF-β by non-covalent interactions, and the latent TGF-β binding protein (LTBP), which is covalently bound to the TGF-β propeptides. Using HT1080 human fibrosarcoma cells as a model for the secretion and proteolytic release of pericellualr matrix-associated TGF-β (Taipale et al.,1992; Taipale et al 1994), we have shown that the ECR3E construct, which consists only of the domain that is bound to the TGF-β propeptide plus flanking EGF-like repeats, binds to TGF-β,and that TGF-β in this complex is not incorporated into the matrix (Mazzieri, unpublished resuts). We have also shown that over expressing the ERC3E results in excess latent TGF-β activation in the skin, and we proposed to characterize its activities when overexpressed in the breast.

Construction of the WAP-ECR3E transgene.

The WAP promoter construct was obtained from a plasmid clone of the coding region for the WAP protein and WAP promoter. This construct was obtained from M. Sternlicht, UCSF. The myc tag on the ECR3E fragment that was suggested in the initial proposal was modified and replaced with hemagglutinin (HA) epitope tag. The reason for this change was the difficulty to detect the myc tag in the skin of K14 - ECR3E myc trasgenic animals (Mazzieri, unpublished data). We designed -

Primers CGGGGATCCACTAGTGGATGTGAATGAATGTGAACT and AACAAGCACT-GCAGTTTCACAG that specifically amplified ECR3E fragment from human LTBP1 cDNA, adding at the same time two HA tags and a stop codon at the the end of ECR3E. The ECR3E-2HA construct was checked after ligation into pBluescript for proper reading frame. The Forward primer was fused to the 3' end of the signal sequence BM40 (SPARC) in the vector pRC/CMV (gift from Rupert Timpl). The ECR3E-2HA fragment was isolated by restriction digestion with the enzymes Asp 718 and BamHI and inserted after the WAP promoter region to replace the WAP coding sequence. The polyadenylation signal was that of the native WAP gene and was contained in the 3' region of the WAP plasmid clone. (Fig. 1). The 4.8 kb fragment obtained after this ligation represented the WAP-ECR3E-2HA construct. The construct was amplified, and the WAP-ECR3E-2HA construct released from the vector by the restriction digestion with EcoRI and purified. The isolated DNA was microinjected into mouse zygotes from the FVB strain. 316 eggs were microinjected with the construct

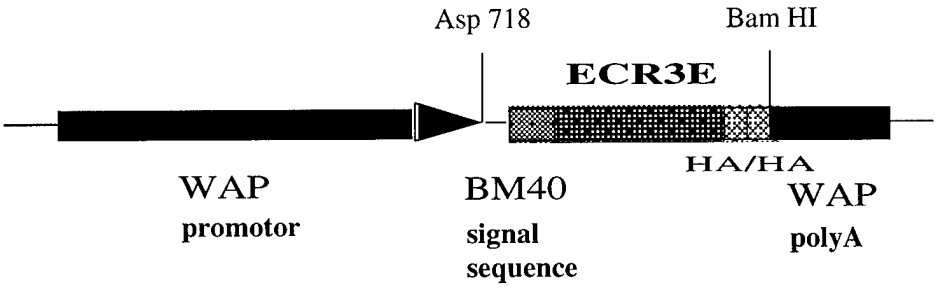

Fig. 1

and 285 were implanted into 9 pseudopregnant females. 42 mice were recovered. The animals were genotyped with specific primers. These span 310 bp corresponding to the region between the 3' end of the WAP promoter (WAP primer: 5' GTAGCCCATCTAGAGCTGTGCC 3') and the 5' end of the ECR3E-2HA fragment (ECR3E primer: 5' CGTTTTCACAGAAGGCTTCACC 3'). We have obtained 9 transgenic animals. These animals are now being bred to monitor transmission of the transgene.

The second construct, which we proposed to make, MMTV-ECR3E presented us with several problems. The pMMTV plasmid, containing the complete, hormone-inducible MMTV LTR promotor / enhancer, obtained from H. Moses, Vanderbilt University, did not have convenient restriction sites for the insertion of the ECR3E. An attempt was made to insert the ECR3E fragment using blunt end ligation. However, this failed even though more than 150 bacterial colonies were screened. In all cases in which the ECR3E-2HA fragment had integrated, the orientation was reversed relative to the MMTV promoter. The reason for this failure to obtain integration in the correct orientation is not clear. We have checked many different bacterial strains with the hope that this might overcame the problem, but we have been unsuccessful. We have started two new approaches. In one we are using special adaptors that will insure directional cloning, and in the other we are creating new restriction sites that will insure the correct orientation.

We altered two aspects of our strategy. First, our intention is to mate the transgenic mice we produced with mice that have been genetically modified so that all females get breast tumors. We had proposed to use were the MMTV-pYMT#121 and MT#634 strains (Guy et al., 1992), but the originators of these mice lost them, e.g. the investigators stopped breeding the mice. However, we have found MMTV-neu #202 strain mice that are commercially available (The Jackson Laboratories) and which have similar properties to the animals we proposed to use as that they have 100%

breast cancer penetrance at 140 days. These mice have been obtained, and we will begin to expand their number so that we can cross them with the transgenic animals. Second, we have not checked for the expression of the WAP-ECR3E transgene in cultured cells. Although WAP is the major whey protein expressed in mammary glands in response to lactogenic hormones, little or no WAP expression is detectable in cell cuture. (Shoenenberg et al., 1990, Park et al., 1989). Therefore we will check for the expression of WAP-ECR3E in the mammary glands of transgenic females in all WAP-ECR3E mouse lines. We will chose the lines with the highest expression of the transgene for breading with MMTV-neu #202 mice and further studies on breast cancer formation.

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Key research accomplishments:

- Creation of WAP-ECR3E-2HA transgene.
- Creation of WAP-ECR3E-2HA transgenic mice.

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